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Survival of *Rhizobium* in Acid Soils

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A *Rhizobium* strain nodulating cowpeas did not decline in abundance after it was added to sterile soils at pH 6.9 and 4.4, and the numbers fell slowly in nonsterile soils at pH 5.5 and 4.1. A strain of *R. phaseoli* grew when added to sterile soils at pH 6.7 and 6.9; it maintained large, stable populations in soils of pH 4.4, 5.5, and 6.0, but the numbers fell markedly and then reached a stable population size in sterile soils at pH 4.3 and 4.4. The abundance of *R. phaseoli* added to nonsterile soils with pH values of 4.3 to 6.7 decreased similarly with time regardless of soil acidity, and the final numbers were less than in the comparable sterile soils. The minimum pH values for the growth of strains of *R. meliloti* in liquid media ranged from 5.3 to 5.9. Two *R. meliloti* strains, which differed in acid tolerance for growth in culture, did not differ in numbers or decline when added to sterile soils at pH 4.8, 5.2, and 6.3. The population size of these two strains was reduced after they were introduced into nonsterile soils at pH 4.8, 5.4, and 6.4, and the number of survivors was related to the soil pH. The *R. meliloti* strain that was more acid sensitive in culture declined more readily in sterile soil at pH 4.6 than did the less sensitive strain, and only the former strain was eliminated from nonsterile soil at pH 4.8; however, the less sensitive strain also survived better in limed soil. The cell density of the two *R. meliloti* strains was increased in pH 6.4 soil in the presence of growing alfalfa. The decline and elimination of the tolerant, but not the sensitive, strain was delayed in soil at pH 4.6 by roots of growing alfalfa.

Legumes are major sources of protein and energy for both humans and domestic animals, and the legume-*Rhizobium* symbiosis is now the most widely managed agricultural system for biological nitrogen fixation. A large portion of the potentially arable land in many regions of the world is acidic (22), and soil acidity is frequently a major constraint for the cultivation of leguminous crops (16). Understanding the behavior of *Rhizobium* in acid soils is therefore important for successful nodulation, development of the nitrogen-fixing symbiosis, and ultimately crop yield.

Concern with the influence of low pH on *Rhizobium* is not new. Bryan (2) found that *R. meliloti*, *R. trifolii*, and *R. japonicum* were unable to survive for 75 days in soils below pH 5.1, 4.9, and 4.2, respectively. Peterson and Goodding (18) reported that, in soils above pH 5.6, the probability of finding *R. melitoti* in soils increased with pH. Jensen (12) and Rice et al. (19) found 10^5 or more cells of *R. meliloti* per g in soils above pH 6.0, but far fewer in more acid soils. The liming of acid soils is known to enhance markedly the survival of *R. meliloti* (24) and *R. trifolii* (1), whereas Mulder et al. (15) found higher numbers of *R. trifolii* under red clover growing in soil of pH 5.1 than in unplanted, limed soil. Studies also have been made of the sensitivity of *Rhizobium* to acidity in culture (7, 8, 11, 13, 14), but the relationship of growth in liquid media to survival or growth in soil has received little attention (1).

R. meliloti seems to be the species of *Rhizobium* most sensitive to soil acidity (2, 7, 12). However, virtually nothing is known about the differences among strains in tolerance to soil acidity, and little has been reported about the survival of several economically important species of *Rhizobium* in acid soils. Hence, studies were initiated to compare the survival of *R. meliloti*, *R. phaseoli*, and a strain of cowpea rhizobia in acid and limed soils and to determine how survival in soil might be related to growth in culture.

MATERIALS AND METHODS

R. meliloti 411 was obtained from the Cornell University culture collection, *R. meliloti* GH1-1 was isolated from nodules of alfalfa growing in Mardin channery silt loam (pH 4.7), *R. phaseoli* 127K17ST was obtained from Carlos Ramirez-Martinez, and cowpea strain 13B, which was originally isolated from an ex-

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tremely acid soil, was provided by Peter Quilt. *R. phaseoli* 127K17ST is resistant to 1.0 mg of streptomycin ml^{-1}. Antibiotic-tolerant mutants of the first two strains were obtained by inoculating dense, rapidly growing cultures onto yeast extract-mannitol (YEM) agar (25) containing 1.0 mg of streptomycin ml^{-1}. Colonies isolated from plates of this medium were grown in liquid culture and then inoculated onto YEM agar containing erythromycin (50 μg ml^{-1}) and streptomycin. These procedures gave isolates of *R. meliloti* 411 and GH1-1 that were resistant to the two antibiotics; the isolates were designated 411SE1 and GH1-1SE1. The same method was used to obtain an isolate of cowpea strain 13B that was resistant to 100 μg of kanamycin ml^{-1}, and this strain was designated $13B^k$. The mutants thus obtained were inoculated onto the host legume growing in disposable plastic pouches (American Scientific Products Corp., Rochester, N.Y.), and the isolates used were able to infect the host and retained the nitrogen-fixing effectiveness of the respective parent strains. The parent cultures were maintained on YEM agar, and the mutant cultures were maintained on YEM agar supplemented with the appropriate antibiotics.

Before use, recently collected soil samples were air dried and passed through a 2-mm sieve. The soils used were Mardin channery silt loam, Windsor loamy fine sand, and Howard gravelly loam from New York; Durham sandy loam from North Carolina; and Piarco fine sand and Princes Town clay from Trinidad. The Mardin soil was limed by mixing up to 5 g of $Ca(OH)_2$ kg^{-1} of air-dried soil. Soil pH values were determined on 1:1 soil:water suspensions.

When sterile soil was to be used, samples of air-dried soil were placed in screw-capped glass tubes and exposed to 2.5 or 10 Mrad of gamma radiation from a ^{60}Co source. The higher radiation dose brought about a drop in pH of 0.18 unit in soils below pH 6.0 and a negligible fall in soils of pH values above 6.0.

For studies of survival, an amount of air-dried soil equivalent to 10 g of oven-dried soil was placed into a sterile 160-ml dilution bottle, and the water content was brought to 20% (wt/wt) with sterile distilled water. The inoculum added to these soils was grown at 29°C in YEM broth on a rotary shaker operating at 75 rpm, and cells in the late logarithmic phase were collected by centrifugation at 16,500 × *g* at 10°C, washed three times in a sterile solution of the inorganic salts used in YEM broth, and suspended in fresh solution. A suspension (1.0 ml) of the washed cells was added to the soil. Except as noted, bottles containing inoculated soil were maintained in the dark at 29°C. At intervals, bottles of soil were sampled in duplicate or triplicate, and the entire contents of the bottles were suspended in and diluted with the sterile salts solution to determine the numbers of rhizobia. The counting medium was YEM agar if the inoculum was added to sterile soil or antibiotic-supplemented agar if the inoculum was added to nonsterile soil. The final concentrations of the antibiotics were 100 μg of kanamycin ml^{-1} and 20 μg of cycloheximide ml^{-1} for counting cowpea strain $13B^k$; 1.0 mg of streptomycin ml^{-1}, 50 μg of erythromycin ml^{-1}, 250 μg of cycloheximide ml^{-1}, and 50 μg of nystatin ml^{-1} for counting *R. meliloti* 411SE1 and *GH1-1SE1*; and 1.0 mg of streptomycin ml^{-1} and 250 μg of cycloheximide ml^{-1} for counting *R. phaseoli* 127K17ST. The minimum number of *Rhizobium* in nonsterile soils that could be counted when streptomycin, erythromycin, cycloheximide, and nystatin were incorporated into YEM agar was 15 g^{-1}. To count protozoa, we used the method of Singh (20).

The lowest pH at which a strain grew in solution was established with a defined medium. This value has been called the critical pH (7). The medium was slightly modified from that of Date and Halliday (6). The modifications consisted of increasing the monosodium glutamate concentration to 6.5 mM to enhance the buffering capacity, substituting mannitol for arabinose, and including thiamine and calcium pantothenate each at 1 mg $liter^{-1}$ and biotin at 0.1 mg $liter^{-1}$. The pH of the medium was adjusted with 1.0 N HCl, and the solution was sterilized by passage through 0.22-μm filters (Millipore Corp., Bedford, Mass.). Culture tubes with 7.0 ml of medium were inoculated with 10^3 to 10^4 rhizobia ml^{-1}. This small inoculum ensured about 10 doublings before the population caused a detectable change in pH. To ensure good aeration, we placed the tubes at an angle on a rotary shaker operating at 75 rpm and observed them regularly for turbidity for up to 4 weeks. The critical pH values of all mutants were the same as those of the parent cultures.

Bacterial abundance in the presence of germinating seeds of alfalfa (*Medicago sativa* var. Iroquois) was determined as described above except that the 10 g of nonsterile soil was placed in scintillation vials, and 20 seeds and the appropriate strain of *R. meliloti* were added at the same time. The vials were capped with Parafilm and incubated at 24°C and 65 μEinsteins m^{-2} s^{-1} under Gro-lux wide-spectrum fluorescent lamps. Seedling size was determined as the linear distance between the root tip and the point of leaf initiation.

RESULTS

The rhizobia were added to sterile soils in initial experiments to examine abiotic effects. A comparison of the survival of *R. meliloti* 411, *R. phaseoli* 127K17ST, and cowpea strain $13B^k$ in unlimed (pH 4.4) and limed (pH 6.9) Mardin silt loam is shown in Fig. 1. In the limed soil, the three strains maintained their population densities, which did not vary by more than one order of magnitude in 5 weeks. In the soil at pH 4.4, however, the population of cowpea strain $13B^k$ remained almost unchanged, whereas the densities of *R. meliloti* and *R. phaseoli* declined rapidly and substantially to values generally between 10^5 and 10^6 per g of soil, at which numbers the populations were maintained.

The ability of the cowpea rhizobium to survive in acid conditions was also evident when it was inoculated into nonsterile soils. This strain survived equally well in soils of pH 4.1 and 5.5 (Fig. 2). In each soil, the size of the population decreased only slightly in 3 weeks. Thus, soils of such acid pH values were not detrimental to this cowpea rhizobium.

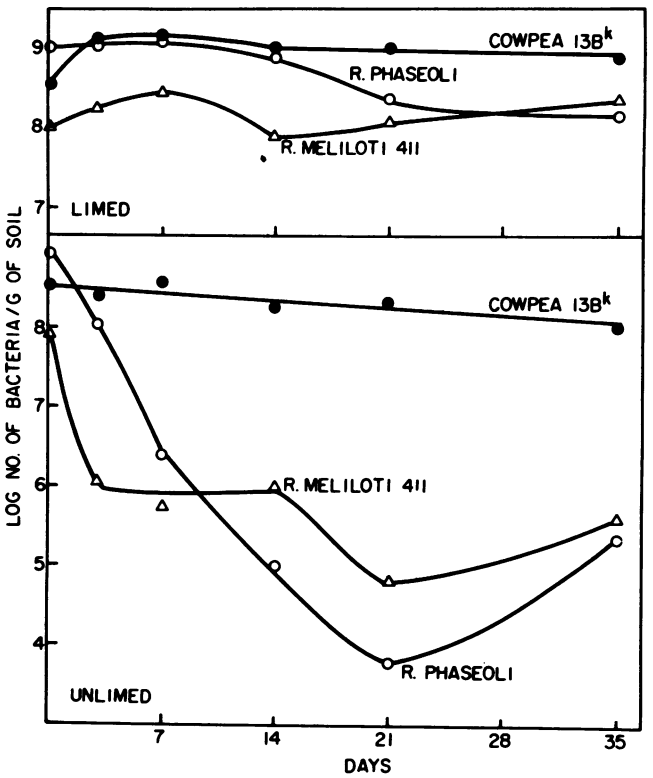

FIG. 1. *Survival of Rhizobium strains added to unlimed and limed sterile Mardin channery silt loam.*

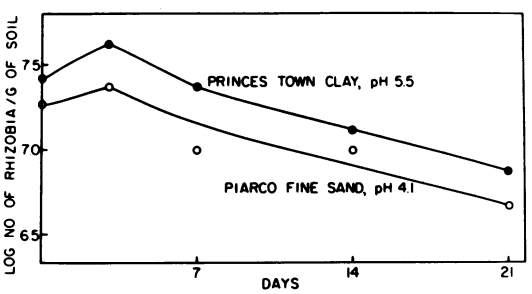

FIG. 2. *Survival of Rhizobium 13B^k in samples of two nonsterile soils.*

The abundance of *R. phaseoli* introduced into samples of sterile soil that had been amended with different amounts of lime was affected by pH. The ultimate size of the population in the sterile soil was increasingly smaller as the pH of the soil decreased (Fig. 3). In the nonsterile soils, however, the population that remained at 3 weeks was essentially the same regardless of the pH. In the nonsterile Mardin silt loam at all pH values tested, the numbers fell markedly within 2 weeks and then leveled off at 10^4 or 10^5 per g. Hence, although this strain of *R. phaseoli* could survive in large numbers when added to sterile soils at high pH values, some factor, which is presumably microbial, led to a marked decline in abundance under nonsterile conditions. Protozoa increased in abundance in these soils after inoculation of *R. phaseoli*, but the protozoan counts among the soils were not significantly different (data not shown).

Before a study of the survival of *R. meliloti*, the growth of strains of this species in acidified liquid medium was evaluated. The mean critical pH value (and standard deviation) for the 10 strains was 5.6 ± 0.2, and the range of values extended from 5.3 to 5.9. Two strains with critical pH values at the extremes of the range were chosen for further study: *R. meliloti* 411SE1 and GH1-1, with critical pH values of 5.9 and 5.3, respectively. For comparison, the critical pH values for *R. phaseoli* strain 127K17ST and cowpea strain 13B^k were 4.4 and 4.2, respectively.

The changes in abundance of *R. meliloti* strains 411SE1 and GH1-1 after inoculation into sterile soils of different pH values are shown in Fig. 4. The behavior of both strains was quite similar in sterile soils with pH values of 6.3, 5.2, and 4.8. In these soils, the numbers decreased initially by one to two orders of magnitude and

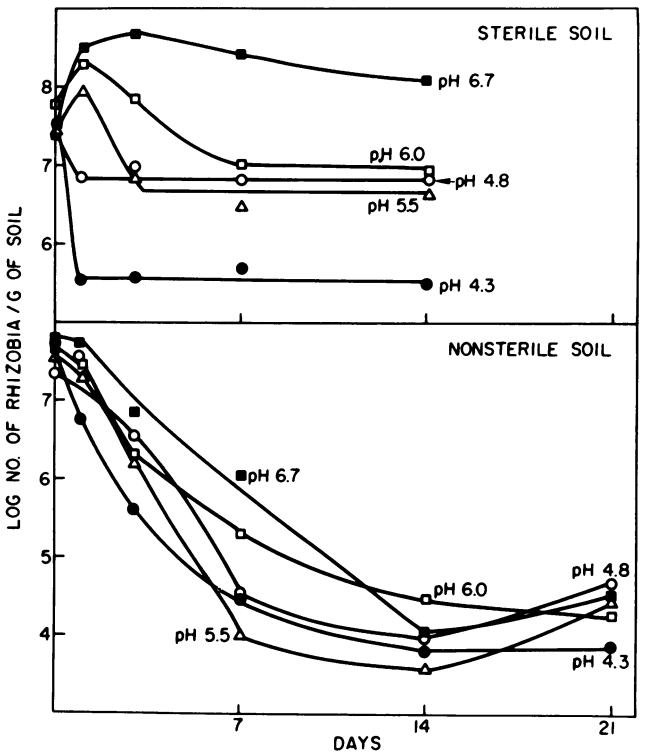

FIG. 3. *Changes in population of R. phaseoli 127K17ST added to sterile and nonsterile Mardin channery silt loam limed to different pH values.*

then increased to approximately the number at day 0. On the other hand, when the two strains were inoculated into sterile Windsor soil of pH 4.6, the initial fall in population was not followed by an increase; moreover, the counts of *R. meliloti* 411SE1 continued to decline and reached 100 g^{-1} of soil at 4 weeks. The population of *R. meliloti* GH1-1, in contrast, dropped to about 1% of the initial value and then fell no further. Thus, survival of the two *R. meliloti* strains differed in the soil at pH 4.6, but not in the other soils.

To assess the population changes of the two *R. meliloti* strains in nonsterile soils, the counts were made on YEM agar supplemented with four antibiotics to suppress the growth of other soil microorganisms. For this purpose, *R. meliloti* strains 411SE1 and GH1-1SE1, which were tolerant to the antibiotics, were added to three soils of differing pH values. The population densities of both strains declined with time (Table 1). Although the initial density of *R. meliloti* GH1-1SE1 was 3-fold greater than that of strain 411SE1, the final numbers of strain GH1-1SE1 were 15- to 60-fold higher at day 30. In the most acid soil (Windsor), strain GH1-1SE1 showed a more rapid initial decline in population density than did 411SE1, but then the counts leveled off, whereas those of 411SE1 continued to fall. A comparison of the behavior of the two strains in Windsor soil (Fig. 4 and Table 1) reveals parallel declines of *R. meliloti* 411SE1 in sterile and nonsterile soil and the leveling off in the decline of *R. meliloti* GH1-1SE1 (or its antibiotic-sensitive parent) in both sterile and nonsterile soils.

A study was carried out to determine whether populations of the two strains of *R. meliloti* were stimulated in acid soils by the presence of germinating seeds of the host legume as reported

TABLE 1. *Survival of R. meliloti GH1-1SE1 and 411SE1 in nonsterile soils*

Soil (pH)	Strain	No. × 10^3 g^{-1} of soil		
		Day 0	Day 14	Day 30
Limed Mardin (6.4)	GH1-1SE1	170,000	16,000	4,200
	411SE1	56,000	1,600	130
Howard (5.4)	GH1-1SE1	170,000	4,200	550
	411SE1	56,000	1,300	37
Windsor (4.8)	GH1-1SE1	170,000	7	6
	411SE1	56,000	240	<1

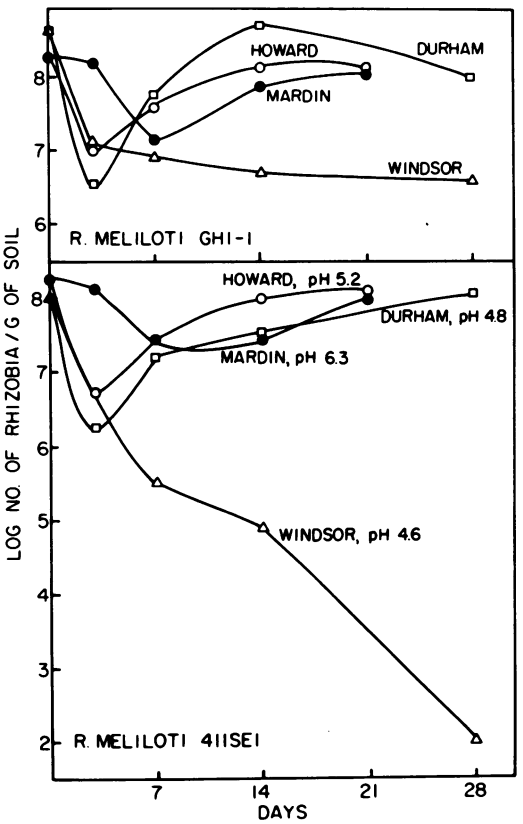

FIG. 4. *Changes in population of R. meliloti GH1-1 and 411SE1 added to sterile Howard gravelly loam, limed Mardin channery silt loam, Durham sandy loam, and Windsor loamy fine sand.*

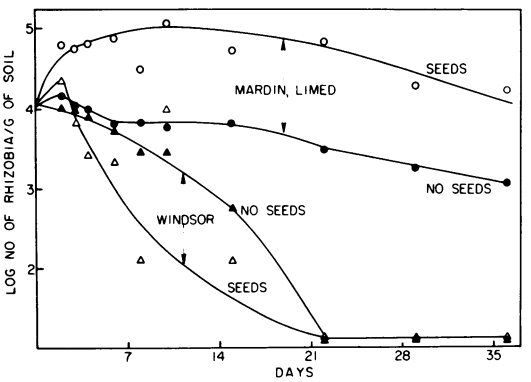

FIG. 5. *Effect of alfalfa seedlings on populations of R. meliloti 411SE1 in limed Mardin channery silt loam and Windsor loamy fine sand. "Seeds" indicates that alfalfa seeds were planted in the soil.*

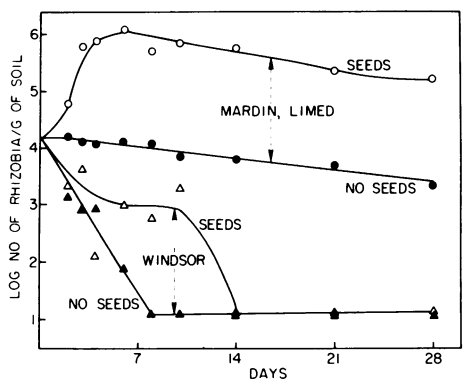

FIG. 6. *Effect of alfalfa seedlings on populations of R. meliloti GH1-1SE1 in limed Mardin channery silt loam and Windsor loamy fine sand. "Seeds" indicates that alfalfa seeds were planted in the soil.*

by Mulder et al. (15). The nonsterile soils were planted with *M. sativa* and inoculated with small numbers of *R. meliloti* GH1-1SE1 or 411SE1. Germination was essentially complete 2 days after planting. In the pH 6.4 soil, the numbers of *R. meliloti* 411SE1 gradually declined from 1.2×10^4 to 1.4×10^3 g^{-1} in 4 weeks in the absence of plants (Fig. 5). In the presence of plants, this strain grew in the first 2 days to a density of 6.3×10^4 g^{-1} and remained at about this level for 3 weeks before falling somewhat in abundance. In the more acid Windsor soil (pH 4.6), on the other hand, the population of *R. meliloti* 411SE1 fell steadily with time until no bacteria could be detected at 3 weeks in either the presence or the absence of plants; the counts in Fig. 5 after 21 days represent the lowest number that could be counted (15 g^{-1}).

R. meliloti GH1-1SE1 behaved similarly to *R. meliloti* 411SE1 in the pH 6.4 soil in the absence of plants, but in the presence of plants, the population was stimulated more markedly than that of strain 411SE1 (Fig. 6). In the pH 4.6 soil, *R. meliloti* GH1-1SE1 fell to undetectable numbers at day 8 in the absence of plants and at day 14 in the presence of plants. Plant growth was similar in the two soils, as indicated by seedling length; therefore, the absence of stimulation of populations of the two *R. meliloti* strains in the Windsor soil in the presence of plants did not result from poorer seedling growth.

DISCUSSION

The results show the striking differences in survival among *R. phaseoli*, *R. meliloti*, and the cowpea *Rhizobium* in acid soils. The number of *R. phaseoli* surviving in sterile soils declined progressively as the pH declined, whereas the cowpea strain was tolerant to a wide range of pH in sterile soil. Because *R. phaseoli* was able to grow in culture at pH 4.4 but exhibited increasingly poor survival in sterile soils of decreasing pH above 4.4, it appears that abiotic factors

other than simply soil acidity limit the ability of this organism to survive. On the other hand, the ability of *R. meliloti* to survive, and even grow, in sterile soil at pH 4.8 is noteworthy because this pH value is lower than the critical pH values of the two strains of this species that were studied. It is likely that the bacteria existed in microsites where they were shielded from the effects of pH of the bulk soil.

The survival of *R. phaseoli* differed in the inoculated nonsterile and sterile soils at all pH values tested. At pH values of 4.8 to 6.7, the cell density never fell below about 10^7 g^{-1} in the sterile soils. In contrast, the density fell to levels of 10^4 to 10^5 g^{-1} in nonsterile soils. This decline may be the result of an attack on the rhizobia by protozoa. Such predation is known to reduce the size of large populations of rhizobia in soil (9). The initial, rapid decline in the inoculated sterile soil at pH 4.3 was not evident in the nonsterile soil; nevertheless, the rhizobia fell to far lower numbers in the nonsterile soil at this pH, the shape of the decline curve being similar to that noted at higher pH values. Because no effect of soil pH on the protozoan response was noted, it is unclear how the abiotic and biotic effects of acid soils combine to reduce the *R. phaseoli* population.

The two *R. meliloti* strains survived equally well in the sterilized, limed Mardin soil and in the Howard soil, but in the nonsterile soils, both survived more poorly in the more acid soil. This suggests a role for biotic factors in the decline of *R. meliloti* in acid soil. These two strains had been chosen for comparison in soils because their critical pH values were different, namely, 5.9 and 5.3. Critical pH values for *R. meliloti* have been reported to be as low as 4.9 (7) and 4.5 (8), but it is not clear whether the isolates of *R. meliloti* were more acid tolerant than the strains used here or whether the differences in results can be attributed to differences in methods. Although the patterns of survival of the two strains in sterile soils above pH 4.8 were nearly identical, the strain with the low critical pH value survived better in all nonsterile soils tested. That some strains of a species of *Rhizobium* survive better than others in soils at different pH values is consistent with the results obtained by Bromfield and Jones (1) in a study of two strains of *R. trifolii*.

Alfalfa increased the population densities of the two *R. meliloti* strains in the limed Mardin soil. In the acid Windsor soil, the decrease in abundance and ultimate elimination of the strain that was more acid tolerant in culture were delayed slightly in the presence of alfalfa, whereas those of the sensitive strain were not. On the other hand, the more tolerant strain declined more quickly in the acid soil. Thus, stimulation by the host plant was not sufficient to overcome the stresses of this acid soil.

The cowpea strain not only was insensitive to changes in soil pH, but it maintained a relatively stable large population in nonsterile soils as compared with other strains used in this study. Such stability in populations in nonsterile soils has been reported for *R. japonicum* (23), *R. leguminosarum* (10), *R. meliloti* (3), and *R. trifolii* (17). Poorer viability in soils was found for *R. meliloti* (5), *R. trifolii* (4), and cowpea rhizobia (5). The differences in behavior among strains of one species suggest that sufficient genetic variability exists to warrant further search for strains with good survivability.

These results confirm the findings of Tuzimura et al. (21) that populations of *Rhizobium* are influenced in complicated ways by soil and plants and that species and strains of *Rhizobium* behave differently. More must be learned about the interaction of these factors if we are successfully to predict the persistence of rhizobia in natural ecosystems.

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